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RESEARCH ARTICLE

ISOLATION AND SCREENING OF BIOSURFACTANT- PRODUCING BACTERIA FROM UYYOKONDAN RIVER, TIRUCHIRAPPALLI DISTRICT, TAMILNADU

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Abstract

Biosurfactants are the compound which helps in reducing surface tension. These compounds help in stabilizing mulsions, promoting foaming, and reducing surface tension etc. The demand for biosurfactants has been steadily increasing and may eventually replace their chemically synthesized counterparts as they are ecofriendly in nature, nontoxic, biodegradable etc. and due to these properties they are used extensively in industries.Biosurfactant producing bacteria were isolated from oil contaminated water samples from uyyokondan river, district of Tamilnadu, South India. The Haemolytic activity, emulsification activity, drop collapsing test as well as oil displacement test were used to determine biosurfactant producing activity of isolated bacterial strains. Among 9 different strains, were isolated and identified by morphologically and 16S rRNA Sequencing as a *Achromobacter denitrificans, Bacillus flexus, Achromobacter xylosoxidans, Bacillus cereus, Pseudomonas medocina, Pseudomonas putida, Bacillus badius, Lysinibacillus xylanilyticus,* and *Exiguobacterium homiense* (99% homology).Strain *Psuedomonas putida* showed the highest activity for oil displacement test and emulsification test. In the present study an attempt on the significant role of bacteria in the production of biosufactant was made.

Introduction:

Oil pollution and remediation technology has become a global phenomenon of increasing importance. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms have an important role in combating environmental pollution. Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size which are surface active compounds which increases the surface tension of hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekedemir, *et al.,* 1999). Almost all surfactants currently produced are chemically derived from petroleum .These synthetic surfactants are usually toxic themselves and are hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have in recent years drawn much attention to the microbial production of surfactants or biosurfactants (Urum and Pekdemir, 2004).

Biosurfactants are a structurally diverse group of surface- active molecules synthesized by microorganisms (Nitschke and Costa, 2007). These amphiphillic compounds contain a hydrophobic and a hydrophilic moiety and have the ability to reduce interfacial tension between different fluid phases. Biosurfactants derived from living organisms, mainly microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatability, higher substrate selectivity, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries (Lu, *et al*., 2003; Thanomsub *et al.,* 2004). The most commonly isolated biosurfactants are glycolipids, and lipopeptides. They include rhamnolipids released by *Pseudomonas aeuroginosa* (Nitschke *et al.,* 2005), sophrolipids from *Candida* sp (Daverey *et al.,* 2008), as well as surfactin and iturin produced by *Bacillus subtilis* strains (Ahimou *et al.,* 2000). Biosurfactant producing microorganisms belong to different genera including *Arthrobacter* spp., *Bacillus* spp, *Candida* spp., *Clostridium* spp.,*Coreynebacterium* spp.,*Nocardia* spp., *Pseudomonas* spp, *Rhodococcus* spp and more other genera have been reviewed (Bannat *et al.,* 2000).

At present few biosurfactants have been used on an industrial scale due to the lack of cost effective production processes. Therefore the search for biosurfactant producing microorganisms that can be grown economically on industrial scale continues. Against these backdrops, this study was aimed at isolating and screening biosurfactant producing bacteria from oil contaminated soil samples.

Materials and methods:

Sampled:

The study was carried out in Uyyokondan river water resources, Tiruchirappalli District, Tamilnadu. The water samples in uyyokondan river collected and all samples were placed an ice thermoinsulated container (temp inside was not higher than 7^0 C), and brought to a laboratory where they were immediately analysed.

Enrichment and isolation of bacterial isolates:

5 grams of water sample was inoculated in 50 ml LB broth (Sambrook *et al.,* 1989) and incubated at 37ºC for 24 hours. 100 l of O/N grown cultures were spreaded on R2A medium agar plates (Anandraj and Thivakaran, 2010). The plates were then incubated at 37ºC for 24 - 48 hrs. Morphologically different colonies were selected and purified (Shoeb, 2006). The selected bacterial isolates were stored in LB agar slants and kept under refrigerated conditions for further screening. Colonies possessing biosurfactant producing activity were then tested for the presence of surfactant by using haemolytic activity, the qualitative drop collapsing test, and quantitative oil displacement test and emulsification activity.

Biosurfactant activity assays:

Haemolytic Activity: Isolated strains were screened on blood agar plates containing 5% (v/v) human blood andincubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carello, *et al.,* 1996).

Drop Collapsing Test: Two microlitters of mineral oil was added to each well of a 96-well microtiter platelid. The lid was equilibrated for1 hr at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive'+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Yuossef *et al.,* 2004).

Emulsification Measurement: Emulsification activity was measured according to the method of Cooper and Goldenberg, 1987 with a slight modification. To 4 ml of culture supernatant or biosurfactant crude extract (0.5%,w/v), 4 ml of *n*-hexadecane were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

Oil Displacement Test: Fifteen µl of weathered crude oil were placed on the surface of distilled water (40 µl)in a petridish (150 mm in diameter). Then, 10 µl of the culture supernatant were gently put on the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 second as described by Morikawa *et al.*(1993).

Biochemical characterization:

Gram stain, Indole production, Methyl red, Citrate utilization test, Voges –Proskauer test, catalase, oxidase, urease test,starch Hydrolysis test, Nitrate reductase test, caesin Hydrolysis test, Gelatin liquefaction test, coagulase etc were performed with chosen isolate (Sneath *et al.,* 1986)

Identification of Strains:

Genomic DNA was extracted from 1ml of bacterial culture, the culture was pelleted by centrifuging at 12,000rpm for 2 min. The pellet was treated with lysis solution and proteinase k and incubated at 60˚C for 30min. Nucleic acids were precipitated with isopropanol and followed by the ethanol treatment at 10,000 rpm for 10 mins. The pellet was washed with 1 ml of 70% (v/v) ethanol solution and dissolved in 0.1 ml of a TE buffer. The purity and quantity of DNA was examined by UV absorption spectrum and agarose gel electrophoresis. 16SrRNA PCR Amplification was performed with the help of the following primer sets: 16s- 8F (5' – GAGAGTTTGATCCTGGCTCAG-3') and 16s-1495R (5'- CGGCTACCTTGTTACTTC-3'). The following PCR conditions were followed (35 cycles of 3min at 940C,1min at 500C, 2 min at 720C and 2min at 720C) and performed in a thermal cycler (Gradient Mastercycler, Eppenorff, USA).The amplified products were subsequently subjected to gel electrophoresis (Banglore Genei, India), stained with ethidium bromide and documented by gel documentation system.

The amplified 16S rDNA was subjected to agar gel electrophoresis and purified by Qiaquick gel extraction kit (Qiagen,USA). The purified PCR product was sequenced by dideoxy chain termination method using ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction kit as directed in the manufacturer protocol. Sequence reactions were electrophoresed and analysed by ABI Prism 3100 genetic analyser (Applied Biosystems, USA). The sequences were analysed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database project (Altrschul *et al.,* 1990). The BLAST analysis was carried out (National Centre for Biotechnology information) to determine the closest bacterial sequences, the closest bacterial sequences were aligned using the Clustal W program (Shingler *et al.,* 1996). Phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbor –joining method (Saitou *et al.,* 1987).The confirmed sequences were deposited in Genebank for public access. (accession numbers KJ452459, KJ452460, KJ452461, KJ452462,KJ452463, KJ452464, KJ452465, KJ452466, and KJ452467).

Results:

Biochemical analysis of the isolates :

As shown in Table 2, Gram staining revelaed that all the isolates were belongs to Gram –negative except organism-2are Gram positive. Catalase and nitrate reductase showed positive results with ten isolates, only organism-3 and 4 are positive results in starch hydrolysis.

16s rDNA gene sequence:

The isolate were further confirmed by 16s rRNA sequencing . Based on DNA extracts of isolate, (Fig-1), 16S rDNA which amplified by PCR using 35 cycles and primers 16sF and 16sR was got sequence result and listed in Table-2. The bacterial16s rDNA sequences were aligned with Blast search of NCBI databases. The sequence aligned have 99% similarity with *Achromobacter denitrificans, Bacillus flexus, Achromobacter xylosoxidans, Bacillus cereus, Pseudomonas medocina, Pseudomonas putida, Bacillus badius, Lysinibacillus xylanilyticus, Exiguobacterium homiense,* respectively. These results highlight the different group of bacterial genera involved in Biosurfactant production. Many scientists studied the biosurfactant production by various *Pseudomonas* species and by *Bacillus species* (Adriano *et al.,* 2007). It is evident from the study that when environment was contaminated with petroleum and oil components the proportion of hydrocarbon degrading microorganisms increases rapidly. High numbers of certain hydrocarbon degrading microorganisms from an environment implies that those organisms are the active degraders of that environment. The presence of oil degrading organisms in the polluted soil is clear indication that the indigenous microbes were carrying out their metabolic activity. The activities of these microorganisms could be responsible for the bioremediation of the environment.

Table.1. Morphological and biochemical characteristics of bacteria isolated from uyyokondan river.

Features	Org 1	Org 2	Org3	Org4	Org5	Org6	Org7	Org8	Org 9
Gram stain	-	+	-	-	-	-	-	-	-
Shape	cocci	cocci	rod	rod	rod	cocci	rod	cocci	rod
Indole	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-
VP	-	-	+	-	-	-	-	+	-
Citrate	-	-	+	+	-	-	+	+	+
Catalase	-	-	-	-	-	-	-	-	-
Oxidase	-	+	-	-	+	+	-	-	-
Urease	+	+	-	-	+	+	-	+	-
Starch Hydrolysis	-	-	+	+	-	-	-	-	-
NR	+	+	+	+	+	+	+	+	+
Caesin Hydrolysis	-	+	-	-	-	+	-	-	+
Coagulase	+	-	+	+	-	-	+	-	+
Gelatinase	+	+	+	-	+	+	-	+	-

Org-organism; +ve- positive; -ve – negative

Table 2: Identification of bacteria by 16s r DNA sequencing

Organism	Sample Source	Identified by 16s sequencing
1	Water	*Achromobacter denitrificans*
2	Water	*Bacillus flexus*
3	Water	*Achromobacter xylooxidans*
4	Water	*Bacillus cereus*
5	Water	*Pseudomonas medocina*
6	Water	*Pseudomonas putida*
7	Water	*Bacillus badius*
8	Water	*Lysinibacillusxylanilyticus*
9	Water	*Exiguobacteriumhomiense*

Fig: 1. Phylogenetic tree showing the relationship among the nine isolates

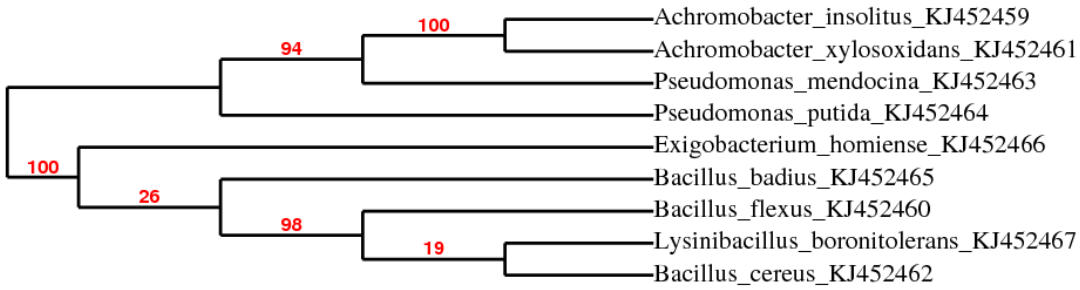

Table.3. Screening results of Nine isolates for the production of biosurfactant

Test isolate	Haemolytic assay	Methylene blue agar plate	Drop collapsing test	Emulsification index	Rhamnolipid (mg/ml)
1	+	+	+	65.2	0.82
2	+	+	+	56.2	0.68
3	+	+	+	50.1	0.56
4	+	+	+	54.8	0.64
5	+	+	+	49.2	0.44
6	+	+	+	71.0	0.89
7	+	+	+	42.1	0.34
8	+	+	+	45.2	0.38
9	+	+	+	47.1	0.59

Screening of production of Biosurfactant:

The nine strains were screened for biosurfatant production test like Haemolytic assay, Methylene blue agar plate, Emulsification index and production of Rhamnolipid. The nine strains are positive to Haemolytic assay, Methylene blue agar plate, Drop collapsing test. The emulsification index and production of Rhamnolipid was highest in *Pseudomonas Putida*.

Discussion:

Most researchers have used maximum two to three screening methods before selecting biosurfactant producers. It is suggested that a single method is not suitable to identify all types of biosurfactants (Yousef *et al.,* 2004). Therefore, a combination of various methods is required for effective screening. Occurrence of biosurfactant-producing bacteria in hydrocarbon-polluted environments was reported by many researchers (Yateem *et al*., 2002; Bodour *et al.,* 2003; Das and Mukherjee, 2005), considering which we selected the Uyyakondan river water for the sampling and isolation of biosurfactant-producing bacteria. Kiran *et al*., (2010) suggested that the single screening method is unsuitable for identifying all types of biosurfactants, and recommended that more than one screening method should be included during primary screening to identify potential biosurfactant producers. In the present study, nine bacterial species positive for biosurfactant production were isolated from river water sample by plate and dilution technique. They were further screened for biosurfactant activities by haemolytic assay, methylene blue agar plate, drop collapsing test, emulsification index, rhamnolipid as reported by satbute *et al*., 2008 that more than one screening method should be included in the primary screening as to identify potential biosurfactant producer (Table 3). The results on methylene blue agar media were similar to the work done by Mulligan *et al.,* (1984) and Mulligan *et al.,* (1989), who have isolated the biosurfactant over producer mutants with methylene blue agar media. The flat appearance in microtitre plate confirmed the positive result for drop collapse test as suggested by Jain *et al.,* (1991) .The result of this research work is in line with those of Tabatabaee *et al*., (2005) and Jaysree *et al*., (2011) who confirmed the isolation of biosurfactant producing microorganisms from oil polluted samples. Also that biosurfactant producing micro organisms can displace oil on water oil interface and show β- haemolysis on blood agar.Our study reports the isolation and subsequent characterization of new biosurfactant-producing isolates from the river water sample.

Conclusion:

Application of biosurfactant and biosurfactant producing bacteria in environmental cleaning (bioremediation) is a potential area of more research as revealed from the present study. Both organic and inorganic contaminants can be removed through different processes (physico –chemical and biological) in which biosurfactants are involved. Due to their, biodegrading potential and low hazard to the environment and human health. These microbes are very promising for use in environmental biotechnologies. The commercial success of such technologies. The commercial success of such technologies is still limited by their high production cost. Nevertheless, careful and controlled use of these interesting surface active molecules will surely help in the enhanced clean up of the toxic environmental pollutants, and provide us with a clean environment.

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